Exploring the parameters of post-segregational killing using heterologous expression of secreted toxin barnase and antitoxin barstar in an *Escherichia coli* case study

Dorien S. Coray,1,2 Brigitta Kurenbach,1,2 and Jack A. Heinemann1,2,*

**Abstract**

Post-segregational killing (PSK) is a phenotype determined by plasmids using a toxin and an antitoxin gene pair. Loss of the genes depletes the cell’s reserve of antitoxin and allows the toxin to act upon the cell. PSK benefits mobile elements when it increases reproductive success relative to other mobile competitors. A side effect of PSK is that plasmids become refractory to displacement from the cell during growth as a monoculture. Most PSK systems use a cytoplasmic toxin, but the external toxins of bacteriocins also have a PSK-like effect. It may be that any toxin and antitoxin gene pair can demonstrate PSK when it is on a plasmid. The secreted ribonuclease barnase and its protein inhibitor barstar have features in common with PSK modules, though their native context is chromosomal. We hypothesized that their recruitment to a plasmid could produce an emergent PSK phenotype. Others had shown that secreted barnase could exert a lethal effect on susceptible bacteria similarly to bacteriocins. However, barnase toxicity did not occur under the conditions tested, suggesting that barnase is toxic to neighbouring cells only under very specific conditions. Bacteriocins are only produced under some conditions, and some conditionality on toxin function or release may be advantageous in general to PSKs with external toxins because it would prevent killing of potential plasmid-naive hosts. Too much conditionality, however, would limit how advantageous the gene pair was to mobile elements, making the genes unlikely to be recruited as a PSK system.

**INTRODUCTION**

Some genes in bacteria are essential for basic cellular functions in all environments. They are maintained in populations because the cell would die without them. Other genes can also benefit from a synthetic indispensability. For example, antibiotic resistance modules are essential in the presence of antibiotics in the environment [1], and antitoxin genes can become essential by neutralizing the effect of a toxin [2, 3]. Modules containing antitoxin genes linked to their cognate toxin become essential in all environments by simultaneously poisoning and curing the cell.

When the toxin and antitoxin genes are paired on plasmids, they may cause an effect called post-segregational killing (PSK). PSK systems are also known as addiction systems [4] because they are difficult to lose once acquired, rendering themselves essential. When toxin–antitoxin (TA) genes are lost, for example, by missegregation during cell division or through plasmid competition [5, 6], the antitoxin is no longer able to protect the cell from the toxin, resulting in bacteriostasis or cell death. This can be because the antitoxin is degraded more rapidly than the toxin [7, 8] or simply because the ability of the antitoxin to counteract the toxin is reduced as both are diluted in the process of cell division after gene loss [9]. Intracellular lability of the antitoxin alone is less likely to be the determining factor in systems where the toxin is continually produced by neighbouring cells.

PSK was first observed when plasmids with TA systems had an apparently greater stability in monoculture. However, PSK plasmids confer a cost on their host and are easily outcompeted by non-PSK plasmids when co-cultured, making this stability an artefact of monoculture [10]. The PSK plasmids are only advantaged under conditions of plasmid competition during horizontal gene transfer [1, 10, 11]. If incompatible, i.e. related, plasmids infect the same host they cannot either replicate or faithfully segregate, and eventually they segregate to homoplasm in different daughter lineages. This also causes any linked TA gene pairs to segregate. The host cells not receiving the PSK plasmid cannot renew the antitoxin and die, and the competitor plasmid is purified from the population.

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**Keywords:** horizontal gene transfer; post-segregational killing; addiction; plasmid competition; barnase; toxins.

**Abbreviations:** PSK, post-segregational killing; TA, toxin–antitoxin.
Most gene pairs that cause PSK described to date consist of a cytoplasmic toxin and an antitoxin that are produced within the bacterium, with the phenotype triggered upon plasmid loss. This includes the well-studied TA systems [4, 12–14] and restriction modification systems [3, 15, 16]. TA systems have labile antitoxins of RNA or proteins, with the toxin targeting membranes and a range of intracellular targets [17, 18]. Restriction modification systems have toxins that cleave DNA and antitoxins that protect the DNA with methylation [9, 15]. They are believed to depend primarily on dilution of both factors [9]. More recently, PSK has been described for abortive infection systems [19, 20] and bacteriocins, which are unique in relying on a secreted toxin [21].

Bacteriocins are a group of narrow-spectrum bacteriocidal proteins, including nucleases and pore-forming proteins [22]. They are organized in an operon with an immunity function and, in most cases, a gene that encodes a cell lysis function to release the toxin into the environment [23]. *Escherichia coli* bacteriocins are exclusively plasmid borne [22], although some closely related bacteriocins are not [22, 24, 25].

Bacteriocins are most often described as bacterial competition systems. Yet, by targeting bacteria closely related to the producing strain, bacteriocins effectively eliminate kin cells that do not have the plasmid. This serves to maintain the plasmid in the population by removing competing cells that have lost, or never had, the plasmid. This resembles a PSK phenotype on a community level [21]. Recently, Inglis et al. [21] found that both a nuclease and a pore-forming colicin were sufficient to stabilize a plasmid in monoculture over 180 generations, even under conditions where toxin expression was low. Cells that lost the plasmid were no longer immune to the toxin and thus were susceptible to residual levels of colicin in the environment.

In conditions where competition is a strong selective force, genes with the potential to cause PSK are recruited onto plasmids. They may also migrate from plasmids to chromosomes, especially when that provides host immunity to the lethal effects of plasmid competition [1]. Indeed, PSK modules are widespread across phyla and replicons [17, 18]. Their ability to render themselves essential is well suited to successful infectious replication.

What features of secreted toxin and internal antitoxin pairs are necessary for their activities to be addictive and provide a benefit to mobile elements? Is PSK a general quality to be expected of secreted toxins and their antitoxins, and could such genes cause PSK simply by moving onto a plasmid? Answers to these questions could help to identify the essential features of PSK evolution and add precision to predictions of what gene activities are likely to be selected on mobile elements and spread across bacteria.

We investigated the ability of barnase, a ribonuclease secreted from *Bacillus amyloliquefaciens*, and its protein inhibitor barstar to confer a PSK phenotype upon plasmids when expressed in *E. coli*. Barnase secreted from *E. coli* using the PhoA signal sequence had been reported to limit the growth of neighbouring bacteria from a wide range of species [26]. Plasmids containing both genes have previously been described as relatively stable over short time periods [27]. These two observations are consistent with some PSK activities. We show that, while barnase is a potent toxin inside the cell expressing it, it is not toxic to the neighbouring cells in a range of conditions, making PSK unlikely to arise just from linkage on a conjugative plasmid. We discuss the necessity of careful regulation of toxicity for such a toxin to act in an effective PSK system.

**METHODS**

**Bacteria and plasmids**

Bacteria and plasmids used in this study are listed in Table 1. *E. coli* strains CSH100 and CSH104 differ in their *lacI* alleles: CSH100 has the *lacI* variant that greatly reduces background transcription from the *P_{lac}*, the initial work was done using plasmid pBB01, containing barstar (*yrdf*) under a constitutive promoter and barnase (*bar*) under *P_{lac}*. This plasmid was only stable in *lacI* genotypes due to toxicity from residual barnase expression. Plasmid pBB05 was derived by amplifying the region downstream of *P_{lac}* and inserting it into a *P_{BAD} TOPO* expression vector (Invitrogen) using restriction sites of *Pmol* and *Nol*. The region from *araC* to barstar was inserted into the *HindIII* and *ClaI* sites of expression vector pJet (Thermo Fisher). An additional barstar downstream of *P_{lac}*, was synthesized (pBS01) and the sequence was inserted into the *Xhol* cut site upstream of the *araC* control gene, to allow for differential control of barstar and barnase. Bacteria were unable to grow without constitutive barstar expression even when cultured under conditions where *P_{BAD}* was repressed. The <*P_{lac}-yrdf araC P_{BAD}-phoA-bar P_{con}-yrdf*> construct was synthesized and inserted into the Cm<sup>6</sup> region of temperature-sensitive plasmid pTS to derive pTS_BB by Integrated DNA Technologies.

**Media**

Liquid and solid media were supplemented with antibiotics at the following concentrations: 100 µg ml<sup>−1</sup> ampicillin, 40 µg ml<sup>−1</sup> kanamycin and 20 µg ml<sup>−1</sup> chloramphenicol. Media were supplemented with 1 mM IPTG and 0.2–2 % L-arabinose to induce expression from *P_{lac}* and *P_{BAD}*, respectively. As specified, cells were grown in LB medium (LB Lennox; Invitrogen), Davis minimal broth without dextrose (M9) or RM medium (M9 broth with 2 % casamino acids). M9 and RM were supplemented with 0.2 % glucose (v/v) or glycerol, and M9 was further supplemented with 100 mg l<sup>−1</sup> thiamine. *E. coli* was primarily cultured at 37 °C; strains containing temperature-sensitive constructs were grown at 30 °C unless otherwise stated.

**Protein extraction**

Samples of CSH100 containing pBB01 were collected to confirm the presence of intracellular and extracellular barnase after induction. Cultures (grown to saturation) were diluted 100-fold in fresh LB then grown to an OD<sub>600</sub> of ~0.500 before exposure to IPTG. Samples (5 ml) were...
Table 1. Bacteria and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Genotype/Description</th>
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<tr>
<td><strong>Bacteria</strong></td>
<td></td>
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<tr>
<td>CSH100</td>
<td>ara-600 (gpt-lac)5 F’ lacFlacZp-4008</td>
<td>[48]</td>
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<tr>
<td>CSH104</td>
<td>ara-600 (gpt-lac)5 F’ lac373 lacZ574</td>
<td>[48]</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pBB01</td>
<td>P&lt;sub&gt;bar&lt;/sub&gt;-phoA-bar P&lt;sub&gt;yrdF&lt;/sub&gt;UC19 backbone. (Amp&lt;sup&gt;§&lt;/sup&gt;) Originally pMT415</td>
<td>[28]</td>
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<td>pBB05</td>
<td>P&lt;sub&gt;bar&lt;/sub&gt;-yrdF araC P&lt;sub&gt;BAD&lt;/sub&gt;-phoA-bar P&lt;sub&gt;yrdF&lt;/sub&gt;UC19 backbone. (Amp&lt;sup&gt;‡&lt;/sup&gt;)</td>
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<td>pBS01</td>
<td>P&lt;sub&gt;bar&lt;/sub&gt;-yrdF, pIDT-SMART backbone. (Amp&lt;sup&gt;§&lt;/sup&gt;)</td>
<td>Synthesized by Integrated DNA Technologies</td>
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<td>pTS</td>
<td>Temperature sensitive, pSC101 derived replica. (Amp&lt;sup&gt;§&lt;/sup&gt;, Cm&lt;sup&gt;‡&lt;/sup&gt;, Kan&lt;sup&gt;†&lt;/sup&gt;) Originally pHSG415</td>
<td>[49]</td>
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<td>pTS_PSK</td>
<td>paeR7, pTS backbone. (Amp&lt;sup&gt;§&lt;/sup&gt;, Cm&lt;sup&gt;‡&lt;/sup&gt;, Kan&lt;sup&gt;†&lt;/sup&gt;) Originally pTN9</td>
<td>[3]</td>
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<td>pTS_BB</td>
<td>P&lt;sub&gt;bar&lt;/sub&gt;-yrdF araC P&lt;sub&gt;BAD&lt;/sub&gt;-phoA-bar P&lt;sub&gt;yrdF&lt;/sub&gt;, pTS backbone. (Amp&lt;sup&gt;§&lt;/sup&gt;, Kan&lt;sup&gt;†&lt;/sup&gt;)</td>
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*Amp<sup>§</sup>, Ampicillin resistance.

‡Cm<sup>‡</sup>, Chloramphenicol resistance.

†Kan<sup>†</sup>, Kanamycin resistance.

§P<sub>con</sub>, Native constitutive promoter for barstar.

Centrifuged to separate cell pellet from supernatant. Cell pellets were lysed by 1 min of sonication. Supernatant (4 ml) was filter sterilized (0.22 µm) and proteins were precipitated with 20% TCA. Protein pellets were washed with cold acetone. Proteins were separated on 4–20% Tris/glycine gels using SDS-PAGE. Total protein was normalized between lanes and stained with Coomassie blue.

**Barstar protection**

Cultures of CSH100 containing pBB05 were grown to saturation in LB (Amp±0.1 mM IPTG) to induce barstar expression. Saturated cultures were diluted 100-fold into fresh LB under the same conditions. Cells were grown for 1 h at 37°C at 180 r.p.m. (OD<sub>600</sub>~1.00) with aeration. New medium was inoculated with ca. 5 × 10<sup>6</sup> c.f.u. ml<sup>−1</sup>. IPTG and/or 0.2% arabinose were added as appropriate. Titres were determined by plating dilutions of samples in duplicate immediately (T<sub>0</sub>) and after 2, 4, 6 and 8 h and enumeration of c.f.u. after incubation at 37°C.

**Temperature-dependent PSK assays**

Early log-phase cultures of CSH104 containing pTS, pTS_PSK and pTS_BB were harvested, washed, diluted 100-fold in antibiotic-free medium and grown at a temperature non-permissive for plasmid replication (42°C) for 6 h. Cells were diluted 100-fold every 2 h to maintain the culture in log phase (below OD<sub>600</sub>~1.00). Cultures were sampled before dilution and plated on LB, ±antibiotics. Plates were incubated at 30°C, and c.f.u. enumerated the following day. Results were taken as the proportion of plasmid-containing cells to all cells.

**Zone of inhibition from secretion of barnase with barstar expression**

Cultures of CSH104 containing pBB05 were grown to saturation in IPTG to induce barstar expression before 10 µl was spotted onto LB, RM and M9 plates with glycerol, IPTG and ±Ara to induce barnase secretion into the medium. Plates were incubated at 37 or 30°C. Experiments were repeated with CSH100 containing pBB01, without barstar induction. After 24 h (LB and RM media) or 48 h (M9 medium), the spots were overlaid with a mid-log phase culture of the same strain (CSH104 or CSH100) without the plasmid. Plates were incubated for a further 24–48 h. Lawns of plasmid-naive strains were also spotted with supernatant from barnase-expressing cells (as extracted above) and lysed whole culture.

**Statistical analysis**

Variation was measured as standard deviation. P-values were generated using two-tailed Student t-tests assuming unequal variance at the final time point of culturing assays.

**RESULTS**

**Development of a non-lethal barnase expression and secretion system in E. coli**

The effect of barnase expression on neighbouring conspecific E. coli was tested using an allele composed of a barnase ORF fused to a PhoA secretion signal [28], to facilitate secretion. Proteins secreted using PhoA [29] are sent to the periplasm but can end up in the medium at high expression levels [30, 31]. While some report problems with saturation of the translocation machinery, inclusion body formation and proteins becoming embedded in the inner membrane.
PhoA has been a successful part of barnase expression platforms since its discovery and early use in protein folding studies [28, 33].

To confirm expression, phoA-barnase was induced and total protein was isolated from cells (Fig. 1a) and the culture supernatant (Fig. 1b), and separated using SDS-PAGE. The band in the cell fraction (Fig. 1a, arrow) correlated with the size of mature barnase (110 amino acids, 12.4 kDa) without PhoA (21 amino acids, ~2.3 kDa) which is cleaved during export into the periplasm [28, 34]. Barnase accumulated after induction in the cells and the supernatant (Fig. 1). At least some of the barnase in the supernatant was likely to have come from dead cells. This is not unlike bacteriocins, which are typically released into the environment via cell lysis. Overall, these results confirmed expression of barnase and its presence in the media.

Despite the expression of constitutive barstar, extended induction of barnase caused growth arrest. We devised several improvements to increase host viability. First, barnase was placed under the control of the $P_{BAD}$ promoter, which is responsive to repression [35]. In addition, an inducible barstar allele was added to the existing constitutively expressed barstar allele (Fig. 2). Both $P_{tac}$ and $P_{BAD}$ promoters are on-off switches [35, 36]. Reducing the inducer (IPTG, arabinose) concentration reduces expression of proteins on a population level, not an individual level [37]. Inducers were used at high concentrations to maximize the number of individual cells in the culture that were expressing both proteins [36, 38].

Induction of barnase from pBB05 in medium supplemented with 0.2% arabinose reduced the density $E. coli$ reached in culture; crucially, expression of both proteins at once relieved growth repression ($P=0.02$, Fig. 3). Hence, the construct on pBB05 balanced the effects of barstar and barnase expression in $E. coli$.

Testing whether barnase and barstar confer a PSK phenotype on plasmids

Plasmids demonstrating PSK are more stable in monoculture than plasmids without [1, 3, 21]. This effect is often used as a diagnostic of PSK activity. We did two assays to test for enhanced stability, using the temperature-sensitive plasmid pTS, which cannot be replicated at 42°C [3] as a

![Fig. 1. Protein profile of $E. coli$ expressing barnase under the $P_{tac}$ promoter. $E. coli$ strain CSH100 containing pBB01 or pP01 was cultured to an OD$_{600}$ of ~0.5 before harvesting, with and without induction (1.0 mM IPTG) of barnase. Protein was separated using SDS-PAGE. Protein content was normalized between samples separately in each gel and was analysed for presence of barnase (~12.4 kDa arrowed). (a) Protein extracted from bacteria culture. (b) Proteins precipitated from the culture supernatant.

![Fig. 2. Organization of barstar and barnase genes in construct BB05. The RNase barnase was under control of the $P_{BAD}$ promoter, which can be induced with arabinose at the transcriptional level and repressed by glucose. The signal sequence phoA targets the protein for secretion into the periplasmic space in $E. coli$. The intracellular inhibitor barstar is present in two copies: under the tac promoter and under a constitutive promoter. AraC is a control gene, capable of up-regulating and down-regulating expression from the promoter.](image-url)
cultured at 42°C. During the course of this assay, the cell culture was diluted every 2 h to maintain the culture in log phase (Fig. 4). Barnase but not barstar was induced for this set of experiments, as expression of barstar from the constitutive allele was sufficient to protect the host from barnase expressed from the low copy number vector for 6 h (data not shown). To determine PSK, the ratio of total c.f.u. in the culture (as measured on LB plates) to number of c.f.u. retaining the plasmid (as measured on a selective medium) was determined. The ratio is close to 1 when all bacteria in the culture have the plasmid, indicative of PSK, and it increases over time when plasmid-free cells accumulate, indicative of no PSK. Only the positive control plasmid produced a ratio close to 1 (pTS_PSK, log ratio=0.86); c.f.u. measurements of cultures containing pTS_BB (log ratio=4100) were not significantly different (P>0.05) to cultures containing the no-PSK control (pTS, log ratio=5300). Thus, barstar and barnase did not confer PSK activity on the plasmid (Fig. 4).

As the same construct is also unstable over 80 generations at 37°C [3, 21], a second assay was performed where the cells were diluted every 24 h, to increase the time period in which barnase could accumulate in the medium. Because expression of barnase from the construct pTS_BB can slow culture growth over these longer periods of time (data not shown), cultures were grown with 1.0 mM IPTG to induce more of the antitoxin. The three cultures were grown for 80 generations without selection in three different kinds of media: LB (Fig. 5), minimal medium and minimal medium supplemented with casamino acids (RM, data not shown). The cultures containing pTS_BB failed to reach 80 generations in minimal medium, presumably due to residual toxicity not fully addressed by barstar expression. In neither LB nor RM medium, where 80 generations was reached, was there a discernible PSK effect, with the cultures containing pTS_BB accumulating plasmid-free cells to the same degree as the negative control (Fig. 5) (P>0.05).

Optimizing lethal conditions

For PSK, the toxin must strongly inhibit plasmid-free cells in the absence of antitoxin. Were our culturing conditions appropriate to make E. coli vulnerable to barnase? We explored the various culture conditions to optimize toxicity caused by barnase. A range of different conditions was explored the various culture conditions to optimize toxicity caused by barnase. A range of different conditions was tested. In the first set of experiments, lawns of control E. coli (containing neither the barnase nor barstar genes) were spotted with lysed and unlysed barnase-expressing cells or the supernatant of barnase-expressing bacteria, which had been filter sterilized and treated with TCA to precipitate proteins. No zones of inhibition were apparent in any of these experiments (data not shown).
Our second set of experiments was inspired by Ramos et al. [26], who previously showed antibiosis using a barstar and barnase expression system similar to ours. The barnase-expressing bacteria were first spotted onto plates and allowed to grow for 24–48 h to allow barnase to accumulate in the surrounding medium. The spots were then lightly stamped with filter paper saturated with control bacteria and incubated overnight to test for antibiosis. Our experiments were performed with bacteria harbouring pBB01 (barnase under control of $P_{\text{tac}}$ and constitutive barstar expression) and pBB05 (barnase under the control of $P_{\text{BAD}}$ and barstar under control of $P_{\text{tac}}$). In contrast to Ramos et al. [26], no instance of antibiosis was detected (Fig. 6).

**DISCUSSION**

PSK is a phenotype that confers reproductive advantage upon horizontally mobile plasmids [1, 10, 39]. By killing hosts that have lost the plasmid, genes that cause PSK eliminate related competitors that infect the same host. Most genes that cause PSK described to date involve an internal toxin and a corresponding antitoxin. If the genes are lost, cell death occurs when the pool of antitoxin is diminished. Recently, some *E. coli* bacteriocins, external toxins linked to an immunity function, have been shown to demonstrate the PSK phenotype in monoculture [21]. We hypothesized that PSK was the outcome of any secreted toxin and antitoxin gene pair linked on a plasmid. To test this, we used the antitoxin barstar and the ribonuclease toxin barnase.

We developed a system that maintained host viability when barnase and barstar were expressed and we used it to test for PSK in short-term and long-term culturing experiments, in a variety of media. Despite using an exhaustive combination of conditions, we did not detect a PSK effect. To drive the PSK phenotype, the secreted toxin would need to be expressed at high enough levels to cause harm to neighbouring bacteria, without killing the host. Barnase will kill the producer cell if expressed in large quantities, even though it is secreted from the cytoplasm by the PhoA signal peptide and co-expressed with barstar. To determine the ideal conditions for antibiosis with our construct, we performed a suite of antibiosis assays, varying temperature, media and expression system. In no condition, from 30 to 37°C in minimal to rich media, did we observe zones of growth inhibition around barnase-expressing cells.

Ramos et al. [26] reported that *E. coli* and soil bacteria were susceptible to barnase secreted from *E. coli* with the PhoA signal sequence, with and without barnase induction. Ramos et al. [26] used an analogous expression system [33] to ours, with barnase under the $P_{\text{tac}}$ promoter and two copies of the barstar gene: one transcribed constitutively and another one under the control of the nifA promoter. Similar to this work,
we found that overexpression of barnase was toxic to producing *E. coli* cells. We did not, however, observe toxicity to neighbouring *E. coli* cells. This may be due to sub-optimal titration of the toxin to antitoxin or small differences in the environmental conditions necessary for the effect.

Our expression system used different promoters to that of Ramos et al. [33] and may not have produced enough barnase and/or barstar. The barnase promoter $P_{\text{tac}}$, used by Ramos et al. [33], is stronger than the $P_{\text{BAD}}$ promoter we used [35]. However, it is likely stronger than the uninduced $P_{\text{tac}}$ expression system which still reportedly produced zones of inhibition. Both systems exported barnase from the cytoplasm using the PhoA signal sequence.

Barstar expression levels are also important, as careful titration is necessary between toxin and antitoxin. Levels of toxin sufficient to kill neighbouring cells could kill the producing cell if not enough antitoxin was present. If this occurs in only a small number of plasmid-bearing cells and the toxin was released into the medium from the dead cells, PSK could still be induced on the community level, but if it occurs in a large number of such cells, then the producers containing the plasmid may die before having an effect on the neighbouring, plasmid-free cells. Such genes would be unlikely to be maintained in a population.

It may also be that toxicity of barnase is only seen under a small range of environmental conditions not fully tested in our experiments. While such conditionality is disadvantageous to experimentation, it may be an important strategy for a PSK system based on an external toxin. Internal toxins in traditional PSK systems only kill cells that have had the plasmid but lost it. However, an external toxin would also kill cells that have never had the plasmid, may not contain competitors and are thus potential hosts. Expressing an external toxin may be particularly disadvantageous when the TA-producing plasmid is invading a new population.

Colicins are expressed in a small fraction of cells after exposure to stress, often nutrient depletion and high population density [40, 41]. The toxin is released into the environment upon lysis of the producing cells (a small fraction of the total plasmid-bearing population), killing neighbouring cells lacking the immunity function. By releasing toxin from multiple cells, more toxin is released in the environment at once to maximize killing efficiency. Doing so during times of high population density maximizes the number of plasmid competitors and plasmid-displaced cells killed during times of high stress while allowing potential hosts to survive during times of low stress.

While the parameters may be narrow and the likelihood in that a given toxin and antitoxin gene pair fulfils them may be small, this is not to say that PSK has not or will not evolve within these constraints. Recently, a type III secretion factor, toxic to host eukaryotic cells, has been shown to share features with known TA systems and act as both a toxin and an antitoxin in the expressing bacterium [42]. While they have not been tested for PSK, PSK exerts a strong pressure for maintenance of genes on plasmids, and once acquired by a plasmid, such genes could spread rapidly. Antibiotic resistance genes are models of rapid spread once mobilized. Antibiotics are also external toxins that select for retention of antibiotic resistance genes, which are essentially rendered an immunity function in their presence [1]. Many antibiotic resistance genes were originally chromosomal genes, later recruited to plasmids [43, 44]. Some antibiotic resistance genes that have been mobilized have alternate functions in their original host [44–47]. However, once in the relevant environment (with antibiotics) and in a host where the gene confers resistance, these genes provide a fitness advantage to either host or mobile vector and become an important pathway for resistance.

Genes that become mobilized can potentially spread to new hosts and into new biochemical and genetic contexts. If they confer an antitoxin function in this new environment, they may spread quickly. Regardless of whether the cognate toxin is exogenous or produced internally by expression of a gene, the genes can be maintained and spread by contributing to the fitness of the mobile elements and making them essential to the cell. The important role of context on functional expression of the PSK phenotype makes it difficult to predict what genes will exhibit it, even if they have the biochemical features of a TA such as barstar and barnase. We can do little to predict what genes will become mobilized though we can predict when they will be – that is, when cellular and environmental factors create selective pressures that favour their presence on mobile elements.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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